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PRINCIPAL INVESTIGATOR: Michel M. Sanders, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, Minnesota 55455-2070

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Michel M. Sanders, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota
Minneapolis, Minnesota 55455-2070

E-Mail: sande001@umn.edu

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13. ABSTRACT ***(Maximum 200 Words)***

An observation was made that the mRNAs for two clones, #41 and #44, were rapidly repressed by estrogen in chick oviduct. Clone #44 shared sequence homology with members of the multidrug resistance-associated protein (*mrp*) gene family. This raised the question of whether the multidrug resistance (mdr) phenotype in breast cancer may be in part due to the loss of repression of MRP expression by estrogen when antiestrogens are administered. The goals of this last year were to complete the identification of clone #41 and to publish a manuscript describing the work completed thus far. The latter goal has been accomplished and is included as Appendix A. The results with MRP1 have considerable significance in light of two recent reports that indicate that MRP mRNA is highly expressed in primary breast cancers, particularly those with poor prognosis. Our observations raise the possibility that one or more of the selective estrogen receptor modulators (SERMs) may be useful in treating those resistant cancers. While we do not as yet have the complete sequence of clone #41, about 1,000 new bp of sequence has been obtained. The ~1300 bp in hand encodes a predicted protein with about 96% identity to two human and one mouse partial clones of the kelch superfamily.

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INTRODUCTION (Adapted from 98/99 report)

The overall goal of our research has been to investigate the regulation of eucaryotic gene expression by steroid hormones. Through the use of differential display, we discovered that two mRNAs are rapidly repressed by estrogen in chick oviduct. Sequence analysis demonstrated that one of these shares 73% identity at the amino acid level with the human multidrug resistance-associated protein1 (hMRP1). This protein is a member of the ATP-binding cassette (ABC) transmembrane transporter family. As members of this family are implicated in the development of resistance to therapeutic drugs, the ability to regulate the amount of MRP1 by estrogen selectively in mammary cells may provide a novel means of enhancing the efficacy of chemical interventions for breast cancer. Thus, the hypothesis under investigation is that estrogen directly represses the transcription of the human *MRP1* gene. The original goals of these studies were to determine whether clone #44 is a chick homologue of one of the human *MRP* genes, whether clone #41 represents another yet unidentified member of the multidrug resistance transporter family, whether expression of the human *MRP* homologue is repressed by estrogen, and whether that repression represents an effect on transcription or mRNA stability. Our broader hypothesis is that at least some estrogen-independent breast cancer cells become resistant to chemotherapeutic drugs when estrogen receptor is no longer capable of repressing expression of the *MRP1* gene. These studies have provided the foundation upon which to design experiments that are directly applicable to designing innovative therapies for breast cancer based on manipulating the expression of the *MRP1* gene.

BODY (Adapted from 98/99 report)

TECHNICAL OBJECTIVES:

***Specific Aim I*: To characterize clones #44 an #41 to determine whether they are homologues of the human *MRP* gene family**

***Specific Aim II*: To determine whether the human *MRP1* gene is regulated by estrogen**

***Specific Aim III*: To determine whether the regulation by estrogen is at the level of transcription, and, if so, to begin to define the critical regulatory mechanisms**

RATIONALE, METHODS, AND RESULTS:

Specific Aim I: To characterize clones #44 an #41 to determine whether they are homologues of the human *MRP* gene family

Original Rationale:

Preliminary results suggested that we have cloned two members of the *MRP* transporter family from chick oviduct. Clone #44 appeared to be the orthologue of human *MRP1*. Based on the tissues in which it is expressed, clone #41 did not appear to be human cMOAT (*MRP2*), the only other *MRP* family member cloned at that time [1]. The goal of this Specific Aim was to more completely characterize the chick cDNAs as the basis for extending the observation regarding the regulation by estrogen to the human. As both chick clones were less than 300 bp, the first objective was to obtain longer cDNAs for each so that sequence comparisons could be made.

Task 1: Cloning and sequencing of a larger cDNA for clone #44:

As described in the 98/99 report, this task is complete and a manuscript describing those results was published in 2000 (Appendix A).

Task 2: Cloning and sequencing of the cDNA for clone #41:

This technical objective continues to thwart us as we still do not have a positive identification for this clone. However, we have obtained an additional 1,000 bp of sequence (Appendix B, Figure A). Database searching of even the most recent human and mouse genome databases with this additional sequence has not provided the identity of the clone. Surprisingly, though, significant homology exists between clone #41 and the kelch domains of two hypothetical human proteins and of one mouse testis protein (Appendix B, Figure B). None of these proteins have been identified or their functions determined as of yet. The homology among these proteins covers the entire 1300 bp we have sequenced and is remarkably high, ranging from 96.7 to 97.2% amino acid identity. Thus, we may have the chick orthologue of at least one of these proteins. This is particularly intriguing as other proteins containing kelch repeat domains appear to play fundamental roles in gonadogenesis [2]. In fact, mutations in the founding member of this family, kelch, lead to sterility in *Drosophila* females [3]. While these observations suggest that clone #41 is *not* part of the ABC transporter family as originally hypothesized, they open up a whole new venue of estrogen-regulated genes whose encoded proteins appear to play significant roles in the function of reproductive tissues. Obviously, further investigation is warranted to determine whether the protein encoded by clone #41 plays a phsyiological or pathological function in human mammary gland.

At this time, we have 17 clones that we are excising from phage in the form of plasmids for sequencing. It is our expectation that at least one of these will contain more of the coding sequence of clone #41 so that we can determine whether this is a novel or previously identified kelch family member. Our expectation is that the gene will be orthologous to one of the three genes listed in Appendix B, Figure B and whose partial sequence is already known. However, in contrast to what is known about the human or mouse genes, we know a considerable amount about how the clone #41 gene is regulated. As indicated in previous progress reports, we have already undertaken considerable characterization of the regulation of the gene, so once the sequence is complete, a manuscript can be submitted within a month.

To summarize, Specific Aim I is virtually completed and it is expected that a manuscript will be submitted within the next six months.

Specific Aim II: **To determine whether the human *MRP* gene is regulated by estrogen**

Original Rationale:
As chick MRP mRNA rapidly decreases ($t_{1/2}$ = ~45 min) upon injection of estrogen (Appendix A, Fig. 4), this raised the intriguing possibility that the human gene is also regulated by estrogen. This contention is supported by the observation that human MRP mRNA is markedly reduced in estrogen-responsive tissues such as ovary, brain, and liver [4]. Additional circumstantial evidence comes from the observations that the human breast cancer MCF-7 cell line typically loses functional estrogen receptors as the cells acquire resistance to drugs [5, 6]. The ultimate goal of this specific aim was to determine whether the human *MRP1* gene is regulated by estrogen. If it is, this may explain how at least some estrogen-independent breast cancers become resistant to chemotherapeutic drugs. Because of the difficulties associated with determining whether the *MRP1* gene is regulated by estrogen in humans, these studies will use human breast cancer cell lines, particularly MCF-7 cells.

Task 3: Treatment of MCF-7 cells with estrogen agonists and antagonists:

As indicated in the last report, initial experiments using a quantitative, competitive reverse transcription PCR (RT-PCR) that we developed indicate that human MRP1 mRNA from MCF-7 cells is rapidly degraded in response to estrogen treatment, with a half-life of 2.5 hours. This is somewhat slower than was seen in the oviduct cells, where the half-life is closer to 45 minutes. The basis for this discrepancy is unclear but may be due to 1) different assay methods- QC RT-PCR versus northern blot, 2) different species- human versus chick, or 3) different molecular mechanisms. Nonetheless, estrogen does have a profound effect on the levels of MRP mRNA in human cells as well as in chick. At this time, experiments are planned to test other estrogen agonists and antagonists as proposed in the grant.

Task 4: Treatment of estrogen-resistant MCF-7 cell line derivatives with estrogen agonists and antagonists:

The goal of these studies was to determine the levels of *mrp* gene expression in cell lines that express estrogen receptor but are resistant to estrogen antagonists. These experiments were never begun because we were unable to obtain the appropriate cell lines. Although we requested the LY-2 [7], MCF7/MIII [8], and MCF7/LCCI [8] cells, all of which are ER positive but antiestrogen resistant, the investigators never responded to repeated requests. Thus, we have elected not to pursue this task any further until additional cell lines with comparable characteristics are available. Furthermore, since this proposal was originally written, considerable information has accumulated about how SERMs (selective estrogen receptor modulators) function [9], making the experiments as proposed antiquated.

To summarize, the primary goal of Specific Aim II is completed although minor goals remain.

Specific Aim III: **To determine whether the regulation by estrogen is at the level of transcription, and, if so, to begin to define the critical regulatory mechanisms**

Original Rationale:

The rapid loss of MRP mRNA in the chick oviduct after treatment with estrogen could reflect an effect on the transcription of the gene, on the stability of the resultant mRNA, or on both. Although no one has examined the half-life of MRP mRNA, that for mdr (multidrug resistance) mRNA is 30 - 60 minutes [10], which is consistent with the half-life of MRP mRNA observed in chick. The goal of this task is to determine the molecular basis for the rapid loss of MRP mRNA after treatment with estrogen.

Task 5: Do nuclear run-on assays with nuclei from MCF-7 cells.

To determine whether estrogen is repressing the transcription rate of the *mrp* gene, nuclear run-on assays were done using nuclei isolated from chick oviduct that had been withdrawn or treated with estrogen. The nuclear run-on assays were performed as we have done before [11-13]. This experiment has been done three times using the complete cDNA clone as the probe, and no signal was seen from the *mrp* cDNA slots despite the fact that signals were seen with two transcription factors, δEF1 and HNF3β (data not shown and [12, 13]. This indicates that the level of transcription of the *mrp* gene is very low, even without estrogen, further suggesting that the level of regulation by estrogen is post-transcriptional. Because we were unable to see a signal even in the estrogen-withdrawn oviduct cells, which have high levels of MPR mRNA, these experiments were not repeated in MCF-7 cells, which have relatively lower expression. As indicated in the previous report, the primary oviduct cell culture model was not suitable for approaching this question using actinomycin D to inhibit transcription. This actinomycin D experiment has not as yet been attempted in the MCF-7 cells. In summary, all of our analyses thus far indicate that the effect of estrogen is post-transcriptional. Because estrogen has well-documented effects on mRNA stability, it is likely that estrogen destabilizes MRP mRNA through as yet undefined mechanisms. Furthermore, reports from other labs indicate that MRP mRNA levels are regulated at the post-transcriptional rather than transcriptional level [14], supporting our interpretation.

Task 6: Do pulse chase experiments with MCF-7 cells.

Because results in the chick system indicate that the effects of estrogen are post-transcriptional, we decided not to utilize resources to pursue this task.

Task 7: Create an MRPCAT reporter vector and transfect into MCF-7 cells.

From the results of Task 5, this task became irrelevant.

Task 8: Make and transfect additional MRPCAT reporter constructs:

From the results of Task 5, this task became irrelevant.

Task 9: Investigate DNA-protein binding interactions.

From the results of Task 5, this task became irrelevant.

Task 10: Define the sequences in MRP mRNA that are regulated by estrogen.

Although the results are not completely solid at this point, it seems likely that estrogen modulates the stability of MRP mRNA. Therefore, it is appropriate to pursue this Task.

In summary, the major goal of this specific aim has been accomplished. However, the minor goals remain to be completed.

KEY RESEARCH ACCOMPLISHMENTS

- Obtained an additional 1000 bp of sequence for clone #41
- Identification of clone #41 as encoding a member of the kelch superfamily
- Demonstration that human MRP mRNA is repressed by estrogen
- Determination of the half life of human MRP mRNA in response to estrogen
- Demonstration that the effects of estrogen on *MRP* gene expression are post-transcriptional

REPORTABLE OUTCOMES

- Manuscript published (Appendix A)
- Additional sequence for clone #41
- Postdoctoral position obtained by graduate student (David Monroe) at the Mayo Clinic as a result in part of his research on this project
- Recruitment of an MD/PhD student (Michael Unruh) into the lab as a consequence in part of his interest n the clone #41 project
- One additional manuscript close to completion on the clone #41 project
- Another manuscript expected on the effects of estrogen on expression of the human *MRP1* gene

CONCLUSIONS REGARDING THE ENTIRE PROJECT

(Adapted from the 98/99 report)

Although the research project has not progressed as rapidly as planned, the observation that human MRP mRNA is down-regulated by estrogen with a half life of about 2.5 hours is a key and seminal observation. This result takes on new significance with the recent observations that MRP mRNA is present in 70% of breast cancer tissues, that its level of expression is significantly elevated in cancerous versus noncancerous breast tissue, and that it is much higher in patients that relapse ten years after chemotherapy [15, 16]. Furthermore, this increase is specific for MRP as MDR gene expression is not increased in primary breast cancer. Thus, one of the major hypotheses underlying the proposed research has been verified, albeit by other groups. Our major contribution to this field is the observation that estrogen opposes MRP by rapidly promoting the loss of MRP mRNA, presumably by destabilizing it. Thus, these observations are highly significant as they demonstrate for the first time that MRP may be useful as a marker for poor prognosis in patients with breast cancer. Furthermore, our observations raise the possibility that one or more of the selective estrogen receptor modulators (SERMs) may be useful in treating those resistant cancers.

While our recent observations with clone #41 suggest that it is not a member of the *MRP* gene superfamily, they appear to expand the effects of estrogen into a whole new arena. This contention is based on the fact that clone #41 contains kelch repeat domains, making it a member of the kelch superfamily. This is a family of proteins known to interact extensively with other proteins and to contain some members that are essential for ovarian function, albeit in *Drosophila.* The role of these proteins in mammalian reproductive tissues remains to be determined. Nonetheless, the observation that estrogen causes the loss of clone #41 mRNA

with a half-life of approximately 1 hour in chick oviduct suggests that this protein may play a critical role in vertebrate reproductive tissues. These observations certainly warrant further investigation, and it is our intention to ascertain whether estrogen affects the expression of the human orthologue in human mammary tissue as soon as we have obtained the chick and human sequences.

In summary, the studies supported by this grant have provided important information about how estrogen signaling occurs in reproductive tissues. In specific, a hypothesis has been proposed and supported experimentally that would explain how breast cancer cells become resistant to therapeutic drugs. In addition, the expression of at least some members the kelch superfamily have been shown for the first time to be regulated by estrogen. The novelty and importance of these seminal observations will inspire continued research endeavors both by us and by others. It is anticipated that the data acquired through those expanded research efforts will provide new information about the effects of estrogen in the normal or cancerous human mammary gland.

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Appendix A

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Repression of chick multidrug resistance-associated protein 1 (*chMRP1*) gene expression by estrogen

Steven Gene Hagen [a], David G. Monroe [a], Diane M. Dean [b], Michel M. Sanders *

[a] *University of Minnesota, Department of Biochemistry, Molecular Biology and Biophysics, 6-155 Jackson Hall 321 Church St., SE Minneapolis, MN 55455, USA*

[b] *St. Joseph's College, Department of Chemistry, 1678 Asylum Ave., West Hartford, CT 06117, USA*

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Abstract

Although a number of genes have been identified whose transcriptional activities are stimulated by estrogen, relatively few have been discovered that are repressed. In an effort to determine whether estrogen can directly repress gene expression, attempts were made to identify genes that are direct targets of the estrogen receptor and whose activities are repressed by it. Because the development and differentiation of the chick oviduct are exquisitely dependent upon estrogen, this seemed an appropriate model system for testing this hypothesis. RNA was isolated from estrogen-treated and estrogen-withdrawn chick oviducts and was subjected to differential display analysis. Surprisingly, one of the products repressed by estrogen encoded the chick homolog of the multidrug resistance-associated protein 1 (*MRP1*) gene. Further cloning resulted in a chick *MRP1* (*chMRP1*) cDNA clone that is 72% identical with human *MRP1*. Translation of the *chMRP1* sequence indicates a 77% amino acid identity with both the human and mouse MRP1 proteins. Treatment of estrogen-withdrawn chicks with 17β-estradiol decreased chMRP1 mRNA levels to 50% within 30 min and to 70% by 1 h, which is comparable to the level observed with chronic repression by estrogen. ChMRP1 mRNA is present in many other tissues, including the heart, lung, brain, kidney, skeletal muscle, and intestine, but is undetectable in the liver. This study indicates that in estrogen-responsive tissues such as chick oviduct, the regulation of *chMRP1* gene expression is controlled by estrogen.

Keywords: ABC transporters; Differential display; Gene regulation; MRP gene family

1. Introduction

Multidrug resistance (MDR) occurs in many different tumor types and presents an obstacle to the treatment of these tumors (Gottesman and Pastan, 1993). The MDR phenotype is characterized by the resistance of the tumor to chemotherapeutic agents that are structurally and mechanistically unrelated. MDR can occur inherently, as in renal and colon carcinomas, or can be acquired during treatment of the tumor by chemotherapeutic agents as in lymphomas and breast carcinomas (Gottesman and Pastan, 1993). The MDR phenotype is ascribed at least in part to the expression of two gene families, the *MDR* gene family that encodes P-glycoprotein and the *MRP* family that encodes the multidrug resistance-associated proteins (MRP) (Loe et al., 1996a,b). Both P-glycoprotein and MRP belong to the ATP-binding cassette (ABC) transmembrane transporter family and are characterized by multiple membrane-spanning domains and nucleotide-binding domains (Higgins, 1992). It is thought that members of the MDR or MRP families confer drug resistance by transporting chemotherapeutic agents out of cells.

The human *MRP1* gene was cloned and characterized from the small-cell lung-cancer cell line H69AR and was shown to provide multidrug resistance to transfected cells (Cole et al., 1992; Grant et al., 1994). To date, five additional members of the *MRP* gene family (*MRP2-6*) have been identified (Kool et al., 1999a,b and references

Abbreviations: ABC, ATP-binding cassette; bp, base pair; cDNA, complimentary DNA; chMRP1, chicken multidrug resistance-associated protein 1; DD-PCR, differential display polymerase chain reaction; DES, diethylstilbestrol; huMRP1, human multidrug resistance-associated protein 1; kb, kilobase; MDR, multidrug resistance; muMRP1, murine multidrug resistance-associated protein 1.

* Corresponding author. Tel.: +1-612-624-9637; fax: +1-612-625-2163.
E-mail address: sande001@tc.umn.edu (M.M. Sanders)

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within). Initial experiments have suggested that *MRP2-6* may also be involved in conferring the multidrug resistance phenotype since expression of these genes is elevated in cell lines selected for cisplatin and doxorubicin resistance (Kool et al., 1997); however, only *MRP2* and *MRP3* have demonstrated organic anion transport activity (Keppler et al., 1997; Kool et al., 1999b). All of the MRP family members belong to the ABC transporter superfamily. Members of this superfamily are ubiquitous from bacteria to man and transport a variety of molecules including amino acids, sugars, inorganic acids, polysaccharides, peptides, and proteins (Higgins, 1992). While the physiological role of MRP remains largely unknown, it can act as an ATP-dependent transporter of cysteinyl leukotrienes, glutathione disulfide, and steroid conjugates including 17β-estradiol 17-(β-D glucuronide) (Leier et al., 1994; Loe et al., 1996a,b).

Estrogen plays a critical role in the differentiation and development of the chick oviduct. The action of estrogen in regulating oviduct differentiation occurs through nuclear estrogen receptors, which function as ligand-dependent transcription factors (Tsai and O'Malley, 1994). To further understand the mechanism of estrogen-regulated gene expression in the chick oviduct, identification of primary targets of the estrogen receptor is necessary. Using differential display polymerase chain reaction (DD-PCR) (Liang et al., 1994), a fragment of an estrogen-repressed gene showing significant homology to the human *MRP1* gene was cloned. An investigation into the regulation of chick *MRP1* gene expression by estrogen is presented in this report. Understanding the mechanism of estrogen action in regulating *MRP1* gene expression could prove useful in the elucidation of the function of MRP1 in development and differentiation and possibly provide clues as to its regulation in the treatment of tumors.

2. Materials and methods

2.1. Animals

Sexually immature female white leghorn chicks were subcutaneously implanted with two 10 mg diethylstilbestrol (DES) pellets (Hormone Pellet Press, Leawood, KA) for at least 2 weeks to induce the development of the oviduct. Estrogen-stimulated chicks retained the DES pellets while estrogen-withdrawn chicks had the DES pellets removed for 5 days. Acute estrogen stimulation was performed by wing-vein injection of 25 mg/kg 17β-estradiol (dissolved in 95% propylene glycol and 5% ethanol). Injection of this solution without 17β-estradiol had no effect on the expression of chMRP1 mRNA. The magnum portion of the oviduct was removed at 0.5, 1, 2, or 4 h after injection and tissue stored at −70°C until use.

2.2. Differential display polymerase chain reaction (DD-PCR) and cloning of the chMRP1 cDNA

Total RNA was isolated using an RNeasy RNA isolation kit (Qiagen, Valencia, CA). DD-PCR was performed as previously described (Liang and Pardee, 1992; Liang et al., 1994) with a few modifications. Reverse transcription was carried out using oligo $dT_{11}G$, $dT_{11}C$, and $dT_{11}A$ primers in separate reactions. Subsequent amplification was performed using the original oligo $dT_{11}N$ primer and a single random primer (5′-TGACGTACAC-3′). cDNAs were compared between the DES-stimulated and DES-withdrawn RNA samples on a 6% denaturing polyacrylamide sequencing gel. Replicate chicks were used as a source for each time point. Differentially displayed cDNA fragments were excised from the sequencing gel and reamplified using the same primers used in the original PCR reaction. The amplified cDNAs were cloned into a T-tailed Bluescript vector (Stratagene, La Jolla, CA). The Gene Trapper Kit (Gibco-BRL, Rockville, MD) was used to obtain a larger cDNA fragment of the *chMRP1* gene (GenBank Accession No. AF265216) from a cDNA library constructed from estrogen-withdrawn chick oviduct mRNA using a cDNA library kit (Gibco-BRL). The estrogen-withdrawn oviduct cDNA library was screened using the Gene Trapper kit with an oligonucleotide primer (5′-CTGGCCACCCCTATAGCTGC-3′) designed from the differential display cDNA clone. cDNA clones were sequenced on an ABI DNA sequencer in the Institute of Human Genetics Microchemical Facility at the University of Minnesota.

2.3. RNA isolation and Northern blot hybridization

Oviducts were removed from chicks, and the RNA was isolated using RNAzolB (TelTest, Friendsville, TX). Total RNA (20 μg/lane) was used to perform Northern blots as previously described (Chamberlain and Sanders, 1999). The cDNA probe for *chMRP1* used in Northern blot analysis was the 266 bp DD-PCR product. The 18S RNA probe was generated from a mouse 18S RNA clone obtained from American Type Tissue Collection (Rockville, MD).

3. Results

3.1. Partial cloning and sequence of the chick multidrug resistance-associated protein 1 (chMRP1) gene

In an effort to understand the regulation of genes involved in chick oviduct development, differential display polymerase chain reaction (DD-PCR) was used to clone genes whose expression is repressed by estrogen. Sexually immature chicks were implanted with DES, a

synthetic estrogen, pellets for at least 2 weeks prior to DD-PCR analysis to initiate oviduct growth and development (Sanders and McKnight, 1985). After the 2 weeks of initial DES stimulation, the pellets were removed for 5 days from the chicks that are designated as estrogen-withdrawn (W/D). The chicks in which the DES pellets were not removed are designated as estrogen-stimulated (Stim). DD-PCR was performed using total RNA isolated from estrogen-withdrawn and estrogen-stimulated chick oviducts using an oligo $dT_{11}N$ primer and a single random primer. cDNA bands generated by the DD-PCR technique were compared between the estrogen-withdrawn and estrogen-stimulated RNA samples. cDNAs that appeared to be down-regulated by estrogen were isolated, re-amplified, subcloned, and sequenced. Sequence analysis of a 266 base pair DD-PCR clone that was differentially expressed in estrogen-withdrawn and -stimulated oviducts indicated a 73% identity at the nucleotide level with the human multidrug resistance-associated protein 1 (*huMRP1*) gene.

To further confirm that the DD-PCR clone was in fact the chick homolog of *huMRP1*, a larger clone was obtained by screening an estrogen-withdrawn chick oviduct cDNA library (Figs. 1 and 2). Nucleotide and amino acid alignment of the *chMRP1* cDNA clone with the human *MRP1-6* genes indicates the highest homol-

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Nuc                                                                    A.A.
1    CGCGGCCGCGTCGACCAATTCTGGTAATGACTGATGGAGAAATCTCTGAGATGGGCTCCT
       R  P  R  R  P  I  L  V  M  T  D  G  E  I  S  E  M  G  S  Y    20
61   ACCAGGAGCTGCTGAAGCAGGATGGAGCTTTTGCAGAGTTCCTTCGTACGTACGCTAATG
       Q  E  L  L  K  Q  D  G  A  F  A  E  F  L  R  T  Y  A  N  A    40
121  CTGAACAAAGCATGGAGAGCAGTGATGCAAGTAGTCCATCTGGAAAGGAACGAAAGCCCG
       E  Q  S  M  E  S  S  D  A  S  S  P  S  G  K  E  R  K  P  V    60
181  TAGAAAATGGAGTCCTTGTGAATGACGCCCCTGGAAAGCTGATGCATCGGCAGCTCAGTA
       E  N  G  V  L  V  N  D  A  P  G  K  L  M  H  R  Q  L  S  N    80
241  ACTCCTCCACATACAGCAGAGAAACTGGGAAGTCACAGCACCAGAGCAGCACAGCAGAGC
       S  S  T  Y  S  R  E  T  G  K  S  Q  H  Q  S  S  T  A  E  L   100
301  TGCAGAAGCCTCTTGCAGAGAAGAATTCCTGGAAACTGACAGAGGCTGACACAGCAGAGA
       Q  K  P  L  A  E  K  N  S  W  K  L  T  E  A  D  T  A  E  T   120
361  CTGGGAGGGTAAAGGCAACAGTATACTGGGAATACATGAAAGCTATTGGACTCTATATCT
       G  R  V  K  A  T  V  Y  W  E  Y  M  K  A  I  G  L  Y  I  S   140
421  CTTTTCTGAGCGTTTTCCTCTTTATGTGTAACCATATAGCCTCCCTGGCTTCCAACTACT
       F  L  S  V  F  L  F  M  C  N  H  I  A  S  L  A  S  N  Y  W   160
481  GGCTAAGTTTATGGACAGATGATCCGGTTGTCAATGGGACACAGCAGTACACAAATGTCA
       L  S  L  W  T  D  D  P  V  V  N  G  T  Q  Q  Y  T  N  V  R   180
541  GACTGGGAGTATATGGAGCGCTGGGAATTTCTCAAGGTATTGCTGTGTTTGGCTACTCGA
       L  G  V  Y  G  A  L  G  I  S  Q  G  I  A  V  F  G  Y  S  M   200
601  TGGCTGTGTCAATAGGAGGAATATTTGCTTCACGGCACCTGCACCTCGACCTGCTGCACA
       A  V  S  I  G  G  I  F  A  S  R  H  L  H  L  D  L  L  H  N   220
661  ATGTTCTCAGGTCTCCAATGAGTTTCTTTGAACGTACACCCAGTGGAAATTTAGTGAACC
       V  L  R  S  P  M  S  F  F  E  R  T  P  S  G  N  L  V  N  R   240
721  GTTTCTCTAAGGAGATAGATACCATTGACTCTACCATTCCACCAATCATCAAGATGTTCA
       F  S  K  E  I  D  T  I  D  S  T  I  P  P  I  I  K  M  F  M   260
781  TGGGCTCAACATTTAACGTGATTGGGGCTTGTATCATCATTTTGCTGGCCACCCCTATAG
       G  S  T  F  N  V  I  G  A  C  I  I  I  L  L  A  T  P  I  A   280
841  CTGCTGTCGTTATTCCACCTCTGGGACTTGTCTACTTGCTTGTGCAGAGATTTTATGTGG
       A  V  V  I  P  P  L  G  L  V  Y  L  L  V  Q  R  F  Y  V  A   300
901  CCACTTCTCGCCAGCTCAAACGCCTTGAATCTGTTAGTCGTTCTCCTGTGTATTCTCACT
       T  S  R  Q  L  K  R  L  E  S  V  S  R  S  P  V  Y  S  H  F   320
961  TCAATGAGACCCTTCTGGGAGTCAGTGTAATTCGAGCCTTTGAGGAACAGAAACGTTTTA  .
       N  E  T  L  L  G  V  S  V  I  R  A  F  E  E  Q  K  R  F  I   340
1021 TAAAGCAGAATGACATGAAAGTGGATGAAAATCAGAAAGCTTATTACCCAAGCATTGTTG
       K  Q  N  D  M  K  V  D  E  N  Q  K  A  Y  Y  P  S  I  V  A   360
1081 CAAACAGATGGCTGGCAGTACGTCTGGAGTTTGTGGGGAACTGTATTGTTCTCTTTGCAG
       N  R  W  L  A  V  R  L  E  F  V  G  N  C  I  V  L  F  A  A   380
1141 CATTGTTTGCAGTGATTGCACGCAACAAGCTCAGTCCGGGACTGATTGGTCTTTCAGTGT
       L  F  A  V  I  A  R  N  K  L  S  P  G  L  I  G  L  S  V  S   400
1201 CCTATTCGCTGCAGATTACAGCATACTTAAACTGGCTAGTTCGTATGACATCTGATCTGG
       Y  S  L  Q  I  T  A  Y  L  N  W  L  V  R  M  T  S  D  L  E   420
1261 AAACCAACATTGTTGCTGTAGAAAGAGTCAAAGAATATGCTGAAATGGAGAAGGAGGTACAG
       T  N  I  V  A  V  E  R  V  K  E  Y  A  E  M  E  K  E  V  Q   440
```

Fig. 1. Partial cloning of the chicken multidrug resistance-associated protein gene (*chMRP1*). DD-PCR was used to clone a fragment of the *chMRP1* gene that is differentially expressed between estrogen-stimulated chicks and estrogen-withdrawn chicks. Additional sequence of the *chMRP1* gene (GenBank Accession No. AF265216) was cloned using the GeneTrapper kit (LifeTechnologies). The original DD-PCR fragment is underlined. The numbers in the left column and right column correspond to the *chMRP1* nucleotide and amino acid sequence, respectively

```
chMRP1   RPRRPILVMT DGEISEMGSY QELLKQDGAF AEFLRTYANA EQSMESSD.. 48
huMRP1   PQVDV-I--S G-K------- ----AR---- --------ST --EQDAEENG
muMRP1   PQVDV-I--S G-K------- ----DR---- ---------- --DLA-E-D.

chMRP1   ..ASSPSGKE RKPVENGVLV NDAPGKLMHR QLSNSSTYSR ETGKSQHQSS 96
huMRP1   VTGV-GP--- A-QM---M-  T-SA--QLQ- ---S--S--G DI..-R-HN-
muMRP1   ..SV-G---- S------M-  T-TV--HLQ- H-----SH-G D-..--QH--

chMRP1   TAELQKPLAE K.NSWKLTEA DTAETGRVKA TVYWEYMKAI GLYISFLSVF 145
huMRP1   ------AE-K -EET---M-- -K-Q--Q--L S---D----- --F-----I-
muMRP1   I-----AG-. -EET---M-- -K-Q--Q-QL S---N----- --F-T---I-

chMRP1   LFMCNHIASL ASNYWLSLWT DD.PVVNGTQ QYTNVRLGVY GALGISQGIA 194
huMRP1   ------VSA- ---------- --.-I----- EH-K---S-- ----------
muMRP1   --L---VSA- ---------- --P------- ANR-F--S-- -----L--A-

chMRP1   VFGYSMAVSI GGIFASRHLH LDLLHNVLRS PMSFFERTPS GNLVNRFSKE 244
huMRP1   ---------- ---L----C- V----SI--- ---------- ----------
muMRP1   I--------- -------R-- ----Y----- ---------- ----------

chMRP1   IDTIDSTIPP IIKMFMGST- NVIGACIIIL LATPIAAVVI PPLGLVYLLV 294
huMRP1   L--V--M--E V-------L- -------V-- -------II- -----I-FF-
muMRP1   L--V--M--Q V-------L- S----V---- --------I- -------FF-

chMRP1   QRFYVATSRQ LKRLESVSRS PVYSHFNETL LGVSVIRAFE EQKRFIKQND 344
huMRP1   ------S--- ---------- ---------- ---------- ------H---
muMRP1   ------S--- ---------- ---------- ---------- ------H---

chMRP1   MKVDENQKAY YPSIVANRWL AVRLEFVGNC IVLFAALFAV IARNKLSPGL 394
huMRP1   L--------- ---------- -----C---- ---------- -S-HS--A--
muMRP1   L--------- ---------- -----C---- ---------- -S-HS--A--

chMRP1   IGLSVSYSLQ ITAYLNWLVR MTSDLETNIV AVERVKEYAE MEKEVQ     440
huMRP1   V--------- V-T------- -S-EM----- ----L---S- T---AP
muMRP1   V--------- ---------- -S-EM----- ----L---S- T---AP
```

Fig. 2. Alignment of *chMRP1* amino acid sequence to previously cloned *MRP1* genes. The *chMRP1* clone was translated and aligned with the human MRP1 (huMRP1) and murine MRP1 (muMRP1) protein sequences, which are 77% identical to the chMRP1 sequence. Numbers in the right column correspond to the cloned *chMRP1* fragment. The huMRP1 (GenBank Accession No. L05628) and muMRP1 (GenBank Accession No. AF022908) sequences are from amino acids 832–1277 and 832–1272, respectively. Dashes represent identity and periods represent gaps in the sequence.

ogy with *huMRP1* (Table 1). This similarity at both the nucleotide and amino acid level between the *chMRP1* cDNA clone and the human *MRP1* gene suggests that the clone is in fact the chicken homolog of the human *MRP1* gene and is part of the superfamily of ABC transporters.

Table 1
Alignment of the nucleotide (%) and amino acid sequence (% identity/similarity) of *chMRP1* with *huMRP1-6*[a]

	chMRP1	
	Nucleotide	Amino acid
MRP1	72	77/83
MRP2/cMOAT	53	45/55
MRP3	56	50/58
MRP4	41	22/32
MRP5	44	30/43
MRP6	51	43/52

[a] Alignments were performed using the GAP program of GCG (Madison, WI). The GenBank Accession Nos for the *huMRP1-6* are as follows: *huMRP1* (L05628), *huMRP2/cMOAT* (U49248), *huMRP3* (Y17151), *huMRP4* (U83550), *huMRP5* (U83551), and *huMRP6* (U91318)

3.2. Regulation of chMRP1 mRNA by estrogen

To confirm that *chMRP1* is indeed differentially expressed in estrogen-stimulated and estrogen-withdrawn chicks, Northern blot analysis was performed using total RNA isolated from chick oviduct (Fig. 3). RNA isolated from 5 day estrogen-withdrawn chick oviduct shows strong hybridization of the *chMRP1* DD-PCR probe to an mRNA species of approximately 6.6 kb. In contrast, the estrogen-stimulated oviduct lane shows a dramatic decrease (sixfold) in the hybridization signal relative to the estrogen-withdrawn lane. These results confirm that DES represses expression of the *chMRP1* gene in chick oviduct.

To determine whether 17β-estradiol, the physiologically relevant estrogen, also represses *chMRP1* gene

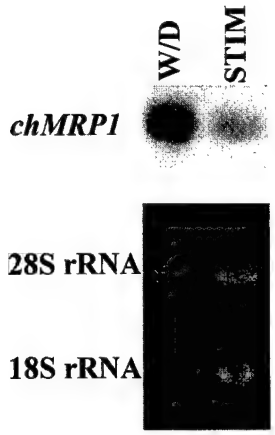
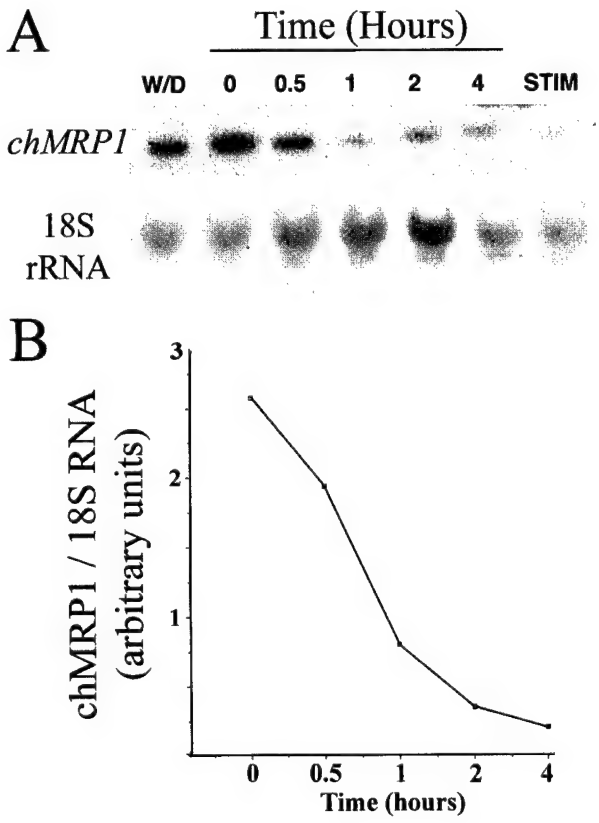


Fig. 3. ChMRP1 mRNA is repressed by estrogen in the chick oviduct. Northern blot analysis was performed using 20 μg of total RNA isolated from estrogen-withdrawn (W/D) and estrogen-stimulated (STIM) oviducts. The 266 bp *chMRP1* differential display cDNA product was used as the probe. The lower panel shows the 28S and 18S rRNA bands stained with ethidium bromide to verify equal loading. The *chMRP1* probe hybridizes to a 6.6 kb band that is sixfold more abundant in the estrogen-withdrawn lane than the estrogen-stimulated lane. This is a representative experiment that was repeated at least three times.

expression and whether it does so acutely, estrogen-withdrawn chicks were injected with 17β-estradiol, and, at the indicated times, the oviducts were removed and



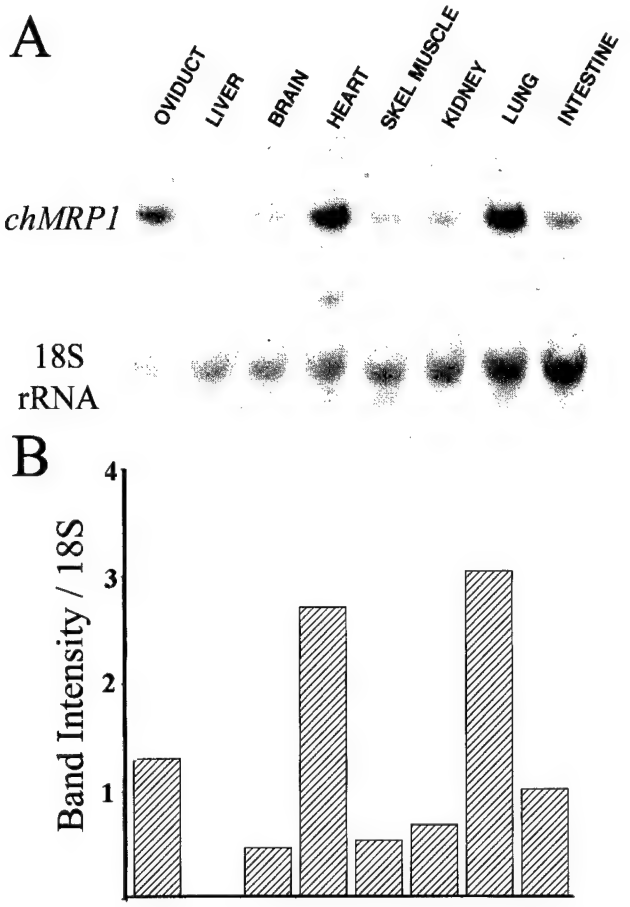


Fig. 5. ChMRP1 gene expression varies between tissues. Tissue distribution of chMRP1 mRNA was determined by Northern blot using tissues from chicks withdrawn from estrogen for five days. (**A**) Northern blot probed with the *chMRP1* clone and an 18S rRNA probe. (**B**) chMRP1 mRNA/18S rRNA values determined by densitometry. The histogram is vertically aligned with the appropriate bands in the Northern blot (Fig. 4A). ChMRP1 mRNA is most abundantly expressed in the oviduct, heart, and lung. The brain, skeletal muscle, kidney, and intestine show lower levels of chMRP1 mRNA expression. ChMRP1 mRNA was not detected in the liver.

RNA isolated. As shown by Northern blot analysis (Fig. 4A), chMRP1 mRNA is abundant in the estrogen-withdrawn oviduct (0 h) but rapidly decreases by 30 min

Fig. 4. ChMRP1 mRNA expression is acutely repressed in the oviduct by estrogen. To determine whether the estrogen-induced decrease in chMRP1 mRNA occurs acutely, chicks that had the DES pellets withdrawn for 5 days were injected intravenously with 25 mg/kg 17β-estradiol. Oviducts were harvested at the indicated times (0, 0.5, 1, 2, and 4 h after injection) or from fully estrogen-stimulated and estrogen-withdrawn chicks, and total RNA was isolated. (**A**) Northern blot analysis was performed using the *chMRP1* clone as a probe, and an 18S rRNA probe was used as a correction for sample loading. (**B**) A plot of the chMRP/18S rRNA values indicates that chMRP1 mRNA decreases rapidly after the injection of 17β-estradiol. ChMRP1 mRNA levels decrease by 50% within 30 min and by 70% within 1 h, which is comparable to levels observed with chronic estrogen administration. This is a representative experiment that was repeated twice.

after injection of estrogen. Densitometric analysis indicates that chMRP1 mRNA levels decrease by 70% within 1 h to a level approximately equal to that observed with chronic DES treatment (Fig. 4B). These results indicate that expression of the *chMRP1* gene is repressed by a natural estrogen in the chick oviduct and that the decrease in chMRP1 mRNA occurs rapidly, within 1 h of estrogen administration.

3.3. Tissue distribution of chMRP1 mRNA

To determine whether chMRP1 mRNA is present in other tissues in the chick, Northern blot analysis was used to determine chMRP1 mRNA levels in estrogen-withdrawn oviduct, liver, brain, heart, kidney, lung, skeletal muscle, and intestine (Fig. 5A and B). The results demonstrate that chMRP1 mRNA is most abundant in oviduct, heart, and lung but is also expressed, albeit at approximately 75% lower levels, in brain, skeletal muscle, kidney, and intestine. ChMRP1 mRNA was not detectable in liver on our Northern blots. These results are comparable to those found with murine MRP1 mRNA, which is abundant in lung, heart, kidney, testes, and muscle and is also expressed in very low levels in the liver (Stride et al., 1996).

4. Discussion

Using the technique of differential display polymerase chain reaction (DD-PCR), a partial clone was isolated corresponding to the chick multidrug resistance associated protein gene 1, *chMRP1* (Fig. 1). Sequence analysis indicates that the *chMRP* cDNA clone is most closely related to the previously cloned human and murine *MRP1* gene (Fig. 2) (Cole et al., 1992; Stride et al., 1996) since nucleotide and protein alignments with *huMRP2-6* indicate a significantly lower homology (Table 1). While the human and murine *MRP1* genes are 88% identical at the amino acid level, the *chMRP1* clone encodes a protein that is 77% identical to human and murine MRP1 (Stride et al., 1996). Structurally, MRP belongs to the family of ATP-binding cassette transmembrane transporter proteins. The members of this family consist of integral membrane ATP-binding proteins that transport a variety of molecules (Higgins, 1992). The region of the *chMRP1* gene that was cloned is located between the two nucleotide-binding domains. Although members of the ABC transmembrane transporter superfamily share structural and functional similarity, identity at the primary sequence level is low, and most of the sequence similarity resides in the nucleotide-binding domains. Within the ABC transmembrane transporter superfamily, MRP1 is most closely related to YCF1 (43% amino acid identity) (Szczypka et al., 1994) but also has sequence identity with the cystic fibrosis transmembrane regulator (19%) (Riordan et al., 1989), the rat β cell sulfonylurea receptor (29%) (Aguilar-Bryan et al., 1995), and ltpgpA (32%) (Papadopoulou et al., 1994). The high degree of identity between the *chMRP1* clone and the human and murine *MRP1* genes indicates that it is indeed the chick *MRP1* homolog rather than another member of the ABC transporter superfamily.

These studies also indicate that expression of the *chMRP1* gene is acutely repressed by estrogen in the chick oviduct. Estrogen is critical for the development and differentiation of the oviduct and is involved in the activation of gene expression in cells that express estrogen receptor. However, control of gene expression and oviduct development may also include the repression of genes by estrogen. As shown in Fig. 4, an early response to the administration of estrogen is the down-regulation of the amount of chMRP1 mRNA. It is tempting to speculate that estrogen down-regulates *chMRP1* gene expression in the oviduct so that intracellular estrogen levels remain high during oviduct development.

Regulation of other members of the ABC transmembrane transporter family by steroid hormones has been shown. Mouse MDR mRNA is increased by estrogen and progesterone in uterine epithelial cells (Arceci et al., 1990; Kuo et al., 1995), and expression of the cystic fibrosis transmembrane regulator gene is also increased by estrogen in uterine epithelial cells (Rochwerger and Buchwald, 1993). However, our results are the first to show repression of a member of the ABC transmembrane transporter family by steroids. The rapid decrease in chMRP1 mRNA by estrogen may be due to a decrease in the stability of the mRNA or a decrease in gene transcription. A previous study demonstrated that following recovery from heat shock, MDR1 mRNA levels decline with a half-life of 60 min (Chin et al., 1990). The authors suggest that the decrease in MDR1 mRNA levels occurs at the level of stability. Based on the similarity between the half-lives MDR1 mRNA and chMRP1 mRNA (Fig. 4), we suggest that mRNA stability could account for the repression by estrogen. This notion is strengthened by the observation that mRNA stability is important in the regulation of MRP1 mRNA levels in some human cancer cell lines (Laurencot et al., 1997). Attempts have been made to examine by nuclear run-on analysis the transcriptional activity of the *chMRP1* gene with and without estrogen, but no signal was detected (data not shown), even though, in the same experiments, we could demonstrate effects of estrogen on the transcriptional activity of the estrogen-inducible transcription factor δEF1 (Chamberlain and Sanders, 1999). This suggests that the transcription rate of the *chMRP1* gene is very low. These results, in combination with the extremely rapid decrease in chMRP1 mRNA, suggest that the primary effect of estrogen may be to destabilize the mRNA.

Our results indicate that expression of the *chMRP1* gene is rapidly repressed by estrogen in the chick oviduct. While there is evidence in cell lines that MRP can confer the MDR phenotype, it is unclear what role MRP plays in the MDR phenotype in tumors (Grant et al., 1994). One study observed low to moderate MRP1 mRNA expression in primary breast carcinomas, indicating that MRP1 may play a role in breast cancer (Dexter et al., 1998). This observation, in combination with our data, provides a compelling suggestion that the regulation of *MRP1* expression by estrogen may have an important role in the physiology of breast cancer, but the nature of this role remains unclear. Determination of the elements required for modulation of *MRP1* gene expression or the mechanisms conferring stability to the MRP1 mRNA would be beneficial in understanding and perhaps treating tumors that exhibit the multidrug resistant phenotype, especially those that are responsive to estrogen.

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APPENDIX B, FIGURE A

Clone #41 Sequence

```
   1  TTTTTTTTTT TTTTTTTCTT TTCCTCTCCC CAAAAAGGCA CAGGnTACAA
  51  TTTTTTATGA CACTTAGGCA GGGAAAGGGC ATTTTCCCAC TTTCGGATTT
 101  ATAAATCAGT CTTGCAGCAG ACCACTGGAT TCATTCATGC CATCATCTTC
 151  TCCTTTTCTT CTCCTCCAAA ACAAATTACA GTTTAAAGCC TTTTTCATTA
 201  TACCTATGAA TGGTCACACA GCCATGATAC GATACTGGCC TGGGCATGGC
 251  TGCTACTCCT GTGATAATGC CCGTTGCAGG ATCATAGCAA AGAATAGTGT
 301  CTGTGGCTTC ACCGTTTTCT CGTCTTCCAC CAAGGATATA GATCTTTCCA
 351  TTACACACAG ACATGCCACA ATTCTCCTGT CTGCTGAACG tTATTCTGTA
 401  CATGCATcCa gtTAgTCCTC AATTGGATCA TAGCAGTATA TTGCCTTGGT
 451  GAGCCCACCA GCAACATAAA TCAgATTGTT TAAAgACaCA GcCGTaAtAC
 501  aTcTTTTtGC aATAGGGATA gtGGcagGGa gCAgCCAaGA ATTAGTgTCA
 551  GGATCATAAG ActGAACCTT gTCAGAACAc GTGTTgTCAT CAGGACCGCC
 601  CCCGATCACA AACAGTTTGC CGACACAGCT GGTGACTGCA GGAGAGCTCA
 651  CGGCTTCCTT GAGGGGAGCC ACCTCTGTCC ATCGATTGGA AAACGAATCG
 701  TAGCACTCCA CGCTGCTGAG GCGGTTTTGC CCATCATACC CTCCAAcGAC
 751  aTACACTTTA CCAAGAAGAA CAGCCATTTT ATGACGCCAT CTGCCTTTAT
 801  TCAAGGAGGC AACTCTGATC CAAATGTTAA GCTGAGAGTT ATAAATCCAT
 851  ACATCCCGAC TATTGATTCT TCCACCTGAA ACAAGGATat cATTCCGTAG
 901  GGCACACAct GCATActCAg ACTtGGTaaa cTctGGAAGT TTGGCCAGtG
 951  ATTTCCATTC TCCtGTtACA GGATCGTAgC ACTCaGTGTa tGgCAAGTTA
1001  AACCcTCCAA CTCTTTCACA GCCTCCAACA ACAACTATCA CCTCAGAATA
1051  ACCAGTTGAT CTGCGTgGCC GAgTCCTGGG GGACATCaTC TCATTGCCAA
1101  GGATATGGTA CcTCCTGGCC TCgTGCAgCA gCTGATAgCA CTCTGGGGAA
1151  TTCTGAATCa gctGGTCCaC cTCCaCagTc TGAACgAAgT agTTGGGGTg
1201  TaaCaACgGG AGCCTGACGT GCGTCAGCAG CTCGTGTAAC ACCGGTCTTC
1251  GCAGCTCGAC CGCCCGGTAC ACCCAGCGCA TGACAGCTTC GAACACCATC
1301  TCTTCCTTCG TGATCACCAG TTCATCGCTG CAGATGTAGT CAATAAGCTC
1351  ATCTTTGCCC AGC
```

Figure A: Nucleotide sequence of Clone #41.

Several cDNA clones were obtained from an estrogen-withdrawn chick oviduct library created under the auspices of this grant. The 300 bp differential display clone #41 fragment was used as the probe. The cDNAs were sequenced and approximately 1000 bp of new sequence was obtained. All of this sequence appears to be in the coding sequence as there are no stop codons in one of the reading frames.

APPENDIX B, FIGURE B

Homology search for Clone #41

Name	% Nucleotide Identity	% Amino Acid Identity
H. sapein hypothetical protein FLJ20059	84	97.2
M musculus adult male testis cDNA	84	96.7
H. sapiens hypothetical protein FLJ22673	84	96.9

Figure B: Proteins that share significant homology to clone #41

The clone #41 sequence shown in Figure A was compared to the most recent human and murine databases in a search for the identity of clone 31. Many homologous proteins were obtained, all of which contained a kelch repeat domain. The three most homologous are indicated above. All of these proteins are homologous to the entire ~1300 nucleotides in the clone #41 sequence, which contains multiple kelch repeats. These data indicate that clone #41 encodes a member of the kelch superfamily of proteins.